Abundance and Tissue Distribution of Selenocysteine-Containing Proteins in the Rat

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ABSTRACT

The form and distribution of selenium (Se) in proteins from selected tissues of the rat were studied by measuring ^{75}Se radioactivity in animals provided for 5 months with [^{75}Se]selenite as the main dietary source of Se. Equilibration of the animals to a constant specific activity of ^{75}Se allowed the measurement of ^{75}Se to be used as a specific elemental assay for Se. Skeletal muscle, liver and blood accounted for 73% of the whole-body Se and 95% of the total Se-dependent glutathione peroxidase activity. Over 80% of the whole-body Se was in protein in the form of the selenoamino acid, selenocysteine. All other forms of Se that were measured accounted for less than 3% of the whole-body Se. The Se in protein was distributed in seven subunit sizes and nine chromatographic forms. The Se in glutathione peroxidase accounted for one-third of the whole-body Se. These results show that the main use of dietary Se, as selenite, in rats is for the synthesis of selenocysteine-containing proteins. Furthermore, the presence of two-thirds of the whole-body Se in nonglutathione peroxidase, selenocysteine-containing proteins suggests that there may be other important mammalian selenoenzymes besides glutathione peroxidase.

INTRODUCTION

The dietary trace element selenium (Se) is essential for rats [1], mice [2], and chicks [3], and many benefits of Se supplementation have been reported in cattle [4], sheep [5], horses [6], and humans [7]. The Se-deficiency symptoms include liver necrosis in rats [1] and mice [2], pancreatic atrophy in poultry [8], white muscle disease in cattle [4] and sheep [5], and cardiomyopathy in humans [7].

Although the bacterial enzymes glycine reductase [9], formate dehydrogenase [10], hydrogenase [11], and nicotinic acid hydroxylase [12] contain essential Se, only one mammalian enzyme, glutathione peroxidase (glutathione:hydrogen

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peroxide oxidoreductase, EC 1.11.1.9), is known to require Se. As in glycine reductase [13], Se in glutathione peroxidase is in the form of selenocysteine [14] in the peptide backbone [15].

Se in mammals has long been known to be associated primarily with protein [16–18]. The ^{75}Se in proteins from rats injected with [^{75}Se]selenite has been partially characterized as a mixture of thiol-labile and tightly bound forms by dialysis against various agents [17]. Several types of small molecular weight Se biomolecules have also been reported in rats injected with [^{75}Se]selenite. These molecules include selenotrisulfides [19], selenocysteine [20, 21], dimethylselenide [22], trimethyl selenonium salts [23] and acid-volatile Se [24].

Recent reports of a rat liver selenocysteine-specific tRNA that is active in protein synthesis [25, 26] are difficult to reconcile with the presence of only one selenocysteine-requiring protein in the rat. It is also hard to explain how glutathione peroxidase deficiency alone can cause the many different Se-deficiency symptoms that have been reported in animals [1–8]. Therefore, this investigation was undertaken to identify the predominant chemical forms of Se in selected rat tissues and to determine the tissue distribution of Se, glutathione peroxidase activity, and Se-containing proteins. Some parts of this research have been presented previously at a scientific meeting [27].

MATERIALS AND METHODS

Isotopic Equilibration

Five male weanling rats (40–60 g) of the Sprague-Dawley strain (Simonsen Laboratories, Gilroy, CA) were maintained on a Se-deficient, Torula yeast-based diet (Teklad Test Diets, Madison, WI) supplemented with 40 IU *dl*-α-tocopheryl acetate/kg [28]. The diet contained less than 0.02 ppm Se. [^{75}Se]Selenious acid (New England Nuclear, Boston, MA) was diluted with unlabeled sodium selenite in 0.5 *M* HCl to a specific activity of 50 mCi/mmol Se. The radiolabeled selenite stock solution was diluted (1:1000) each week to a concentration of 0.2 ppm Se in distilled water that was supplied as drinking water for the 5-month equilibration period. The diet and the drinking water with the ^{75}Se were supplied ad libitum. That the tissues of the rats were at least 98% equilibrated with ^{75}Se could be seen from the weight gain over the equilibration period (50 to 460 g) and from the most conservative estimates of the biological half-life of Se in the rat of 48 to 56 days [29, 30]. The percentage approach to equilibrium was calculated as % equilibration =

$$[1 - (50\ \text{g}/460\ \text{g} \times e^{-(0.693 \times 150\ \text{days}/52\ \text{days})})] \times 100\%$$

The 98.5% calculated in this manner is a conservative estimate since most of the Se in the rat has a biological half-life of 7–14 days [29].

Tissue Sample Preparation

The rats were anesthetized with ether and blood was collected by heart puncture into syringes containing 0.6 ml of 1.5% EDTA and 0.9% NaCl. Plasma, erythrocyte soluble and ghost fractions were prepared by the method of Dodge et al. [31]. The carcass was perfused with 50 ml of ice-cold Hank's balanced salts

containing 0.02% sodium heparin (w/v). The hind leg muscle, liver, kidney, testes, epididymides, lungs, and heart were removed and placed in ice-cold 0.25 *M* sucrose/10 mM Tris-HCl (pH 7.8)/0.1 mM EDTA (Buffer A). The tissues were minced with scissors and homogenized in two volumes of Buffer A with six strokes of a glass–Teflon homogenizer. Heart and hind leg muscle were homogenized by grinding for 90 sec in a microblender.

Sephacryl S-300 Chromatography in Sodium Dodecyl Sulfate (SDS) and Urea

Gel-filtration of ^{75}Se-labeled protein subunits was performed on a 1.5 × 90 cm column of Sephacryl S-300 (Sigma Chemical Co., St. Louis, MO) in 0.2 *M* Tris-phosphate (pH 6.9)/7 *M* urea/5 mM SDS. The 0.5-ml samples were mixed with 0.5 g urea, 0.24 ml of 180 mg SDS/ml, 0.25 ml mercaptoethanol, 25 mg dithioerythritol, and 0.08 ml of 25% triethanolamine (v/v) and heated in boiling water for 10 min. Blue dextran and cresol red dyes were added to each sample as internal markers for the column void and total volumes, respectively. The column was eluted at room temperature at a flow rate of 0.15 ml/min and 1-ml fractions were collected.

The ^{75}Se-labeled subunits were characterized by the apparent partition coefficients of the peak ^{75}Se fractions. The apparent partition coefficient was defined as $K_a = (V_e - V_0)/(V_t - V_0)$, where V_e was the peak ^{75}Se fraction, V_0 was the peak fraction of blue dextran, and V_t was the peak fraction of cresol red. The column was calibrated with the following molecular weight standards: phosphorylase, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; and lysozyme, 14,400 ($r^2 = 0.998$).

All of the clearly visible ^{75}Se peaks were assigned to one of seven groups of partition coefficients. A one-way analysis of variance and pairwise *t* tests between groups showed that the means of all the groups were significantly different ($p < 0.01$, 5–21 observations per group). The mean molecular weights and standard deviations for the seven subunit size groups were calculated from the molecular weight standard curve and the partition coefficient statistics [32].

The contribution from each subunit size group to the total ^{75}Se in a chromatogram was calculated by summing the ^{75}Se in the fractions of those peaks that were completely separated or by nonlinear least-squares decomposition of the more complex peaks. The data were fitted by the method of Marquardt [33], as modified by Tabata and Ito [34], to a function that represented a sum of up to five Gaussian peaks, centered at the mean partition coefficients of the appropriate subunit size groups. The Gaussian equations had the means (peak positions) held constant while the height and width were fitted to the data. The iterative least-squares procedure was continued until the solution converged. The criteria for convergence were a relative change of less than 1% in each of the parameters and a standard deviation less than the ^{75}Se counting background. The area under each component Gaussian was then used to estimate the contribution from each subunit size to the total ^{75}Se in the chromatogram (Fig. 1).

DEAE Sephacel Chromatography in Triton X-100 and Urea

^{75}Se-labeled proteins were chromatographed on DEAE Sephacel (Sigma Chemical Co., St. Louis, MO) equilibrated in 25 mM imidazole-HCl (pH 7.8)/7 M urea/

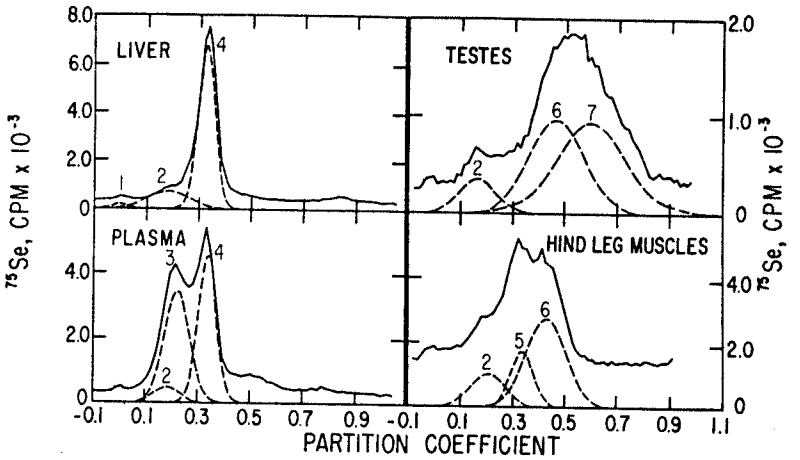

FIGURE 1. Chromatography of whole tissue samples on Sephacryl S-300 in SDS and urea. Dashed lines represent the component Se-containing subunit peaks estimated by non-linear least squares fitting of the data. The numeric labels identify the Se-containing subunits as follows: 1, >89,200 M_r; 2, 46,400 M_r; 3, 36,300 M_r; 4, 26,500 M_r; 5, 20,100 M_r; 6, 14,700 M_r; and 7, 8,400 M_r.

0.1% Triton X-100 (Buffer B). The 0.5-ml samples were mixed with 1 ml of Buffer B, 0.2 ml Triton X-100, 25 mg dithioerythritol, and 0.75 g urea and then heated for 15 min at 50°C. The samples were centrifuged for 10 min at 3600 × *g* and the pellets obtained were twice extracted with 1 ml of Buffer B and centrifuged. The supernatants were combined and adjusted to pH 7.8 with 1 *M* imidazole before they were applied to a 1.5 × 30-cm column of DEAE Sephacel in Buffer B. The column was washed with 60 ml of Buffer B and then eluted with a linear gradient of 0–0.5 *M* NaCl in 300 ml of Buffer B. The column was eluted at room temperature at a flow rate of 0.33 ml/min and 3-ml fractions were collected.

The ^{75}Se peaks were assigned to one of nine groups based on the fraction numbers and conductivities of the peak ^{75}Se fractions (Fig. 2). These groups were designated and are hereafter referred to as chromatographic forms A through I. Chromatographic form I was measured as the ^{75}Se remaining in the top 2 cm of the column packing material after completion of the gradient.

Assays

Glutathione peroxidase was assayed by a modification of the coupled assay procedure of Little and O'Brien [35] with cumene hydroperoxide (30 μg/ml) and glutathione (0.21 mM) as substrates and 10 mM NaCN to inhibit the non-Se enzyme [36]. Glutathione peroxidase enzyme units were defined as nanomoles of NADPH oxidized per minute. The whole animals, major organs, and carcasses were counted for ^{75}Se with a NaI scintillation ratemeter in a shielded enclosure at a distance of 16 in. The amount of Se was calculated by ratio to a long-term ^{75}Se standard prepared from the same ^{75}Se solution used to prepare the drinking water. All other samples were counted for ^{75}Se on a gamma counter (model 5210, Packard Instruments, La Grange, IL) by ratio to a more dilute standard.

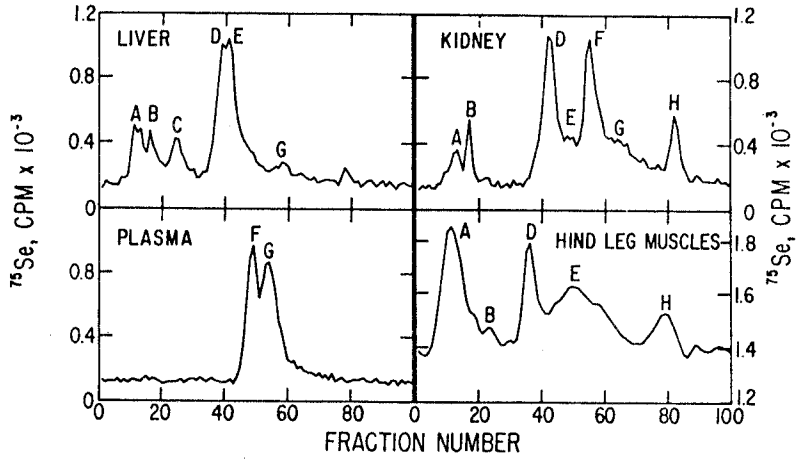

FIGURE 2. Chromatography of whole tissue samples on DEAE Sephacel in Triton X-100 and urea. The letters show the assignment of these ^{75}Se peaks to the Se-containing protein chromatographic form groups. The assignments were based on the elution positions and the conductivities of the peak ^{75}Se fractions (not shown).

Selenocysteine Analysis

Samples of 0.1 ml plasma or 0.5 ml tissue homogenate were mixed with 0.6 g guanidine-HCl/ml and one-half volume of 6 *M* guanidine-HCl/0.15 *M* Tris-HCl (pH 8.0)/15 mM GSH/3 mM dithioerythritol and dissolved by sonication for 5 min. Iodoacetic acid was added to a concentration of 10 mM (3 mM excess iodoacetate over thiol), and the samples were incubated for 10 min at 37°C before adding potassium borohydride to a concentration of 2 mM and incubating for an additional 10 min at 37°C. The samples were then dialyzed against three changes of distilled water and mixed with 0.4 μCi of [^{3}H]carboxymethylselenocysteine recovery marker [37] and one volume of 12 *M* HCl. The samples were flushed with nitrogen, evacuated, and hydrolyzed by heating for 24 hr at 110°C. [^{3}H]Carboxymethylselenocysteine and carboxymethyl[^{75}Se]selenocysteine were isolated on an amino acid analyzer (model 120B, Beckman Instruments, Irvine, CA). Tritium was measured by dual-channel continuous-flow scintillation counting of the eluant and ^{75}Se was counted in 2-min fractions collected at the flow scintillation counter outlet. The ^{75}Se in the fractions at the elution time of carboxymethylselenocysteine (78 min) was summed and divided by the fractional recovery of [^{3}H]carboxymethylselenocysteine to yield the total ^{75}Se as selenocysteine in the tissue samples. Application of this method to partially purified rat liver glutathione peroxidase routinely yielded selenocysteine recoveries of 90%–110% after correction for the [^{3}H]carboxymethylselenocysteine recovery [26, unpublished data]. Other analyses of rat liver glutathione peroxidase by different methods have confirmed that all of the Se is present as selenocysteine [14, 15].

Protein-Bound Selenium

Paired duplicate samples (> 10,000 cpm of ^{75}Se in 0.1–0.5 ml) were diluted as necessary to 0.5 ml with 2 *M* Tris-HCl (pH 8.0). One sample from each pair was

mixed with 1 ml of protease mix (1 mg each of chymotrypsin, trypsin, protease K and Pronase/ml of 2 *M* Tris-HCl) and the other sample from each pair was mixed with 1 ml of 2 *M* Tris-HCl. Pronase was from Calbiochem-Behring, La Jolla, CA. All samples were then incubated for 18 hr at 37°C. Fifty percent trichloroacetic acid was added to a concentration of 10% and the samples were held on ice for 10 min. The precipitates were collected on 0.45 μm Millipore filters, washed twice with 1 ml of cold 10% trichloroacetic acid, and the filtrates and washes were combined. Se released by proteolysis was calculated as the difference in each pair between the ^{75}Se in the filtrate of the protease-treated sample and the ^{75}Se in the filtrate of the control without protease and was expressed as a percentage of the average ^{75}Se in each pair.

Acid Volatile Se

Immediately after the animals were killed, triplicate tissue samples (> 10,000 cpm per sample) were sonicated for 5 min to dissolve 0.9 g guanidine-HCl/ml of sample, mixed with 50 μl of 50 mM Na_2Se, and counted for the initial ^{75}Se content. The samples were placed in 16 × 125 mm stoppered test tubes with 2–18 gauge needles and were flushed with nitrogen for 10 min before being acidified with 12 *M* HCl to a concentration of 3 *M*. The samples were then heated under constant nitrogen purge for 10 min in boiling water. Acid volatile ^{75}Se was calculated as the mean difference within each set of triplicate samples before and after acid and heat treatment.

RESULTS

Biochemical Form of Se

The nine tissues examined in this study accounted for 85% of the whole-body Se, based upon tissue weights from data tables for skeletal muscle, plasma, and erythrocytes [38, 39]. Since these nine tissues accounted for so much of the whole-body Se, an average abundance of a form of Se calculated by weighting the percentage abundance in each tissue by the amount of Se in each tissue is an estimate of the percentage of whole-body-Se in a particular form.

The calculated abundance of selenocysteine in these nine tissues ranged from 56% to 112%, with an average abundance, weighted by the tissue Se [32], of 85% ± 25% (Table 1). This is a minimum estimate of the whole-body abundance of selenocysteine since it is based on 85% of the whole-body Se and because of the possibility of incomplete carboxymethylation by iodoacetate.

From 54% to 92% of the ^{75}Se in the tissue samples was released by treatment with the mixture of proteases, and the average abundance of protein-bound Se (weighted by the tissue Se) was 80 ± 13%. This is a minimum estimate of the whole-body abundance of protein-bound Se since the samples were not denatured prior to protease treatment and since only a single addition of protease was made.

The percentage of tissue Se in small molecular weight forms was estimated by summing the ^{75}Se in the fractions at the column total volume in the chromatograms of tissue samples on Sephacryl S-300 in SDS and urea. These peaks contained not only the native small molecular weight forms of Se but also any Se originally

TABLE 1. Tissue Distribution and Chemical Form of Se in Rat Tissues[a]

Tissue	Weight (g)	Se Concentration (ppm)	Whole-Body Se (%)	Glutathione Peroxidase eu/g[d]	Tissue Se		
					Selenocysteine[b] (%)	Protein Bound[b] (%)	Small Molecular Weight[c] (%)
Muscle	213[e]	0.15	36	2,400	69	92	1.8
Liver	14.4	0.97	16	79,000	112	70	3.1
Plasma	17[e]	0.6	11	270	87	80	1.5
Erythrocytes	11[e]	0.77	9.5	48,000	100	80	2.0
Kidney	3.3	1.6	6.0	19,000	56	62	—[f]
Testes[b]	3.4	1.2	4.4	2,300	113	54	2.0
Epididymides[b]	9.4	0.09	0.95	1,200	61	54	—
Lung	2.6	0.21	0.61	3,100	110	65	3.8
Heart	1.2	0.32	0.43	12,000	102	77	5.2
Average Whole-body Abundance[g]					85 ± 25	80 ± 13	1.9 ± 0.9

[a] Values are the averages of measurements in two animals except as noted.
[b] Single measurements.
[c] Molecular weight $<2000 M_r$.
[d] Nanomoles NADPH oxidized per minute per gram of wet tissue.
[e] Tissue weights from biological data tables [38, 39].
[f] Not detectable.
[g] Weighted by the tissue Se (± S.D.).

present in selenotrisulfide, selenenyl sulfide, or diselenide linkages to larger molecules. The average whole-body abundance of small molecular weight forms of Se (weighted by the tissue Se) was 1.9% ± 0.9%.

Although others have reported the presence of acid volatile ^{75}Se in rat liver after administration of [^{75}Se]selenite [24], there was no significant difference in any of these nine tissues between the ^{75}Se contents before and after acidification and heating ($p \gg 0.1$, paired t test). Other experiments using a nitrogen purge at room temperature also gave negative results. Since the ^{75}Se counting background was < 1% of the count rate in the samples, the steady-state abundance of acid-volatile Se was <1% of the tissue Se.

Samples of liver, kidney, and blood were extracted with chloroform-methanol [40]. These lipid extracts contained 0.62%, 0.12%, and 0.06% of the tissue Se, respectively. No further investigations of these forms were performed because of their very low levels of Se.

Tissue Distribution of Se and Glutathione Peroxidase

Together, skeletal muscle, blood, and liver accounted for 73% of the whole-body Se (Table 1). The highest concentrations of Se were in kidney, testes, and liver, in agreement with the results of Burk et al. [41] and Behne and Wolters [42]. As reported previously [42, 43], the highest concentrations of glutathione peroxidase were in liver, erythrocytes, and kidney. Liver accounted for 50% of the total glutathione peroxidase activity, erythrocytes 23%, and skeletal muscle 22%. A linear regression of the glutathione peroxidase activities against the Se concentrations across all nine tissues from two animals gave a correlation coefficient of 0.19 ($p \gg 0.1$), showing that the enzyme activity was not a good predictor of tissue Se concentrations.

Subunit Sizes of Se-Containing Proteins

The 26,500M_r Se-containing subunit group was shown to include the glutathione peroxidase subunit by cochromatography on Sephacryl S-300 in SDS and urea with ^{75}Se-labeled rat liver glutathione peroxidase purified by published methods [44]. This subunit size accounted for 39% of the whole-body Se and was the predominant Se-containing subunit in liver, plasma, kidney, and heart (Table 2).

The second most abundant Se-containing subunit, 20,100M_r, accounted for 31% of the whole-body Se, with over 80% of that amount in the skeletal muscle. The lack of detectable levels of this protein in liver and testes agrees with a report of a 20,000M_r, non-glutathione peroxidase, Se-containing protein subunit found in rat kidney but not in liver or testes [45].

The 36,300M_r Se-containing subunit accounted for 5.5% of the whole-body Se and was detectable only in plasma, where it accounted for 42% of the plasma Se. This was the only Se-containing subunit that was unique to plasma. An average of only 4.3% of the plasma Se was in the 46,400M_r subunit, which presumably included the plasma Se-containing subunit of approximately the same molecular weight reported by others [18, 46, 47]. However, the 46,400M_r Se-containing subunit was present in greater abundance in every other tissue examined except erythrocytes (Table 2). That the presence of the 46,400M_r Se-containing subunit in

TABLE 2. Molecular Weight Distribution of Se-Containing Protein Subunits in Rat Tissues[a,b]

Tissue	Relative Molecular Weight						
	>89,200	46,400 ±2,300	36,300 ±1,700	26,500 ±1,100	20,100 ±800	14,700 ±900	8,400 ±1,200
Muscle	6.5	18	—[c]	20	55	—	—
Liver	1.3	19	—	75	—	5.4	—
Plasma[d]	0.4	4.3	42	53	—	—	—
Erythrocytes	—	—	—	37	43	20	—
Kidney	—	6.8	—	63	30	—	—
Testes[e]	1.1	8.3	—	—	—	35	55
Epididymides[e]	0.4	4.9	—	—	53	42	—
Lung	—	8.8	—	34	41	16	—
Heart	—	17	—	60	23	—	—
Average Whole-body Abundance[f]	3.1	13	5.5	39	31	5.7	2.8

[a] Molecular weights were measured by chromatography on Sephacryl S-300 in SDS and urea.
[b] Averages for the percentage of tissue Se in two animals.
[c] Not detectable.
[d] Average for three animals.
[e] Single measurements.
[f] Weighted by the tissue Se.

other tissues was not due to plasma contamination could be seen by the lack of detectable levels of the 36,300M_r Se-containing subunit in these tissues.

McConnell et al. [48] reported the presence in testes soluble of a 15,000M_r ^{75}Se-labeled protein that increased in abundance over the course of 21 days after injection of [^{75}Se]selenite. In the present study, a 14,700M_r Se-containing subunit accounted for 35% and 42% of the Se in testes and epididymides, respectively. This subunit size is also in reasonably good agreement with Calvin's estimate [49] of the sperm tail Se-containing protein subunit size of 17,000M_r and with the report of Prohaska et al. [50] of a 16,000M_r Se-containing protein in testes.

The 8400M_r Se-containing subunits were detected only in testes. Their peaks on Sephacryl S-300 in SDS and urea were broader than any of the other Se-containing subunit peaks (Fig. 1), suggesting that there was considerable heterogeneity in the size of these testes Se-containing proteins. As far as we can determine, this is the first report of 8400M_r Se-containing protein subunits in rat testes.

Chromatographic Forms of Se-Containing Proteins

Preliminary investigations of the soluble fractions from liver, kidney, and erythrocytes showed that the ratio of glutathione peroxidase enzyme activity to Se varied over almost a twofold range, suggesting the presence of non-glutathione peroxidase, Se-containing proteins in these soluble fractions as had been reported in rat liver [51]. The major Se-containing proteins in the soluble fractions of erythrocytes, liver, kidney, testes, and plasma were partially purified on 1.5 × 30 cm-columns of DEAE Sephacel in 10 mM Tris-HCl (pH 7.8)/0.1 mM EDTA developed at 4°C with a linear gradient of 0-0.5 M NaCl in 300 ml of the same buffer. The ratio of glutathione peroxidase enzyme units/pmol Se was measured in the peak ^{75}Se fractions.

The major ^{75}Se peaks from the soluble fractions of liver, kidney, and testes eluted before the salt gradient and had glutathione peroxidase specific activities of 8.1, 6.1, and 1.9 enzyme units/pmol Se, respectively. The main ^{75}Se peak from the erythrocyte soluble fraction eluted in the salt gradient at 0.1 M NaCl with a specific activity of 6.6 enzyme units/pmol Se. Pure glutathione peroxidase has a specific activity of 7.5–8.5 enzyme units/pmol Se [14, 44]. The fact that partially purified glutathione peroxidase from liver, kidney, and erythrocytes had specific activities that agreed within ±17% while the ratio of glutathione peroxidase/Se in the tissue homogenates varied by ±58% indicates that some of these tissues contained non-glutathione peroxidase, Se-containing proteins.

The glutathione peroxidase peaks form chromatography of liver, kidney, and erythrocytes on DEAE Sephacel in nondenaturing conditions were each pooled, concentrated, and chromatographed on DEAE Sephacel in Triton X-100 and urea. All three chromatograms contained major peaks of Se at the positions of chromatographic forms B, D, and E, in varying proportions. Since chromatograms of purified rat liver glutathione peroxidase also contained the same three peaks, it is unlikely that these three chromatographic forms represented different physiologically significant forms of the enzyme. The pooled glutathione peroxidase from testes contained too little ^{75}Se to detect when chromatographed on DEAE Sephacel in Triton X-100 and urea.

A single peak of ^{75}Se from plasma (approximately 40% of the plasma Se) eluted at 0.05 M NaCl from DEAE Sephacel under nondenaturing conditions and had no

detectable glutathione peroxidase activity. The rest of the plasma Se remained bound to the column. When plasma Se-containing proteins were chromatographed on columns of Sephacryl S-300 in 10 mM Tris-HCl (pH 7.5)/0.1 mM EDTA, 40% of the plasma Se eluted at 85,000M_r. The rest of the plasma Se remained on the column. Apparently, the 85,000M_r, native Se-containing plasma protein and the one eluting at 0.05 *M* NaCl were the same.

Group I was the most abundant chromatographic form on columns of DEAE Sephacel in Triton X-100 and urea, accounting for 19% fo the whole-body Se (Table 3). Since group I was irreversibly adsorbed to the column, it was probably a complex mixture of acidic and denatured Se-containing proteins, including glutathione peroxidase. Group I was the most abundant chromatographic form of Se-containing protein in plasma, but was present in higher concentrations in kidney and testes.

The highest tissue concentration of group G was in plasma, which contained over two-thirds of the whole-body amount of this chromatographic form. The fact that the 36,300M_r Se-containing protein subunit and group *G* were the only two forms of Se-containing protein that had their highest tissue concentrations in plasma suggests that they both came from the same native plasma protein. Furthermore, their abundances in plasma of 30% and 42%, respectively, imply that they both came from the 85,000 M_r, native Se-containing protein mentioned above.

Chromatograms of plasma Se-containing proteins on DEAE Sephacel in Triton X-100 and urea contained no detectable levels of the glutathione peroxidase chromatographic forms *B*, *D*, or *E*. Even though plasma contained 43% as much of the 26,500M_r Se-containing subunit as liver, plasma had a glutathione peroxidase activity almost 300-fold lower than liver (Table 1). Other investigators have also reported that glutathione peroxidase levels in rat plasma are very much lower than in rat liver [42, 46]. Therefore, the 26,500M_r Se-containing subunit in plasma must have been derived from a non-glutathione peroxidase, Se-containing plasma protein.

DISCUSSION

Biochemical Form of Se

The results of this study clearly demonstrate that the predominant form of Se, given as dietary selenite, in the rat is selenocysteine bound to protein. However, the recent report of a selenomethionine-containing form of bacterial thiolase [52] and the magnitude and standard deviation of the average whole-body selenocysteine abundance, 85% ± 25%, suggest that significant amounts of selenomethionine may have been present. Furthermore, several of the amino acid analyzer chromatograms contained 2% to 5% of the applied ^{75}Se in peaks near the elution positions of selenomethionine and selenocystine (diselenide). Unfortunately, the identities of these peaks could not be established since the amino acid analyzer system did not separate these two selenoamino acids. The abundance and significance of selenomethionine in animal tissues needs to be investigated further.

The most conservative estimate of the average whole-body abundance of protein-bound selenocysteine in the rat would be the product of the selenocysteine abundance and the abundance of protein-bound Se, 0.85 × 0.80 = 0.68, or 68%.

TABLE 3. Distribution of Selenium in Chromatographic Forms on DEAE Sephacel in Triton X-100 and Urea[a,b]

Tissue	Chromatographic Form								
	A	B	C	D	E	F	G	H	I
Muscle	19	4.4	—[c]	14	27	—	—	3.3	28
Liver	4.5	15	4.5	33	11	3.0	1.4	—	5.7
Plasma	—	—	—	—	—	13	30	1.8	40
Erythrocytes	5.9	0.2	13	27	33	0.6	—	—	14
Kidney	2.8	3.4	—	11	6.9	16	6.1	5.1	27
Testes[d]	18	19	10	2.0	1.7	4.2	3.1	1.9	22
Epididymides[d]	1.3	10	5.6	—	24	5.2	—	49	4.1
Lung	6.8	—	1.9	18	—	21	10	—	18
Heart	5.8	4.1	1.6	18	23	11	—	14	22
Average whole-body abundance[e]	11	6.0	2.9	16	18	3.9	4.9	2.6	19

[a] Data from integration of ^{75}Se peaks in chromatrograms of whole tissue samples.
[b] Averages of the percentage of tissue Se in each chromatographic form in two rats, except as noted.
[c] Not detectable.
[d] Single measurements.
[e] Weighted by the tissue Se.

For the following reasons, we conclude that the main use of dietary Se, when provided as selenite, is for the synthesis of selenocysteine-containing proteins. Both of the measurements of protein-bound selenocysteine were prone to underestimation; the only macromolecular forms of Se reported in animals have been proteins; 98% of the Se was associated with high molecular weights; and other forms of Se accounted for less than 3% of the whole-body Se.

Our finding that selenocysteine is the predominant form of Se in rat tissues is in agreement with the results of Beilstein et al. [53], who found that selenocysteine was the major form of ^{75}Se in ovine heart and liver 5–10 days after injection of [^{75}Se]selenite, and with previous results from this laboratory that showed selenocysteine to be the predominant form of ^{75}Se in rat tissues 3 hr to 6 days after injection of [^{75}Se]selenite [54]. The most significant new finding from the present study is that non-glutathione peroxidase, selenocysteine-containing proteins are the predominant steady-state form of Se in the rat without regard to turnover times, biological half-lives or isotope dilution, which limited the interpretation of many earlier studies [16–21, 45, 53, 54].

Even though the selenocysteine in these proteins was not shown to be inside the peptide backbones, the fact that most of the tissue Se existed as selenocysteine, even in the presence of the ubiquitous selenocysteine lyase [55], shows that at least the amino group of selenocysteine was in a stable bond to protein (amide or peptide bond). If the selenocysteine had had a free amino group, the endogenous selenocysteine lyase would have degraded it. The fact that less than 2% of the ^{75}Se was released from protein by treatment with SDS, urea, dithioerythritol, and mercaptoethanol at 100°C for 10 min shows that the binding of selenocysteine to protein was very strong.

Abundance of Glutathione Peroxidase

The mean molecular weight of the 26,500M_r Se containing subunit group was in good agreement with a recent report from this laboratory of a native tetrameric molecular weight of 105,000 for rat liver glutathione peroxidase and a subunit size of 25,000, measured by SDS polyacrylamide gel electrophoresis [44]. Our finding of different native charge forms of glutathione peroxidase in liver and erythrocytes is consistent with similar findings by others for liver [51], lung [56], and erythrocytes [57, 58].

Based upon the weighted average whole-body abundance of the glutathione peroxidase subunit size (26,500, Table 2) and the fact that plasma contained very little glutathione peroxidase activity (Table 1) and no detectable amount of chromatographic forms *B*, *D*, or *E* (Table 3), we estimate that glutathione peroxidase accounted for 39%-(0.53 × 11%) = 33% of the whole-body Se. This is in reasonably good agreement with an estimate based on the sum of the weighted average abundances of the glutathione peroxidase chromatographic forms *B*, *D*, and *E*, or 40% of the whole-body Se. This latter estimate is somewhat high owing to the presence of non-glutathione peroxidase, Se-containing proteins in these chromatograhpic forms in testes and muscle. These estimates both agree well with the estimate by Behne and Wolters' [42] that less than 40% of the whole-body Se in rats was bound to glutathione peroxidase as calculated from neutron activation analysis of Se and the specific activity of glutathione peroxidase.

Heterogeneity of Selenocysteine-Containing Proteins

Although the chromatographic techniques used in this study separated the Se-containing proteins into seven subunit sizes and nine chromatographic forms, there were several indications of heterogeneity within these groups. (i) Group I (irreversibly adsorbed to DEAE Sephacel in Triton X-100 and urea) probably contained all Se-containing proteins with an isoelectric point below some critical pH as well as some denatured proteins. (ii) Similarly, chromatographic form A (at the column void volume) probably contained all Se-containing proteins with an isoelectric point above some critical pH. (iii) Plasma contained large amounts of a non-glutathione peroxidase, 26,500 M_r, Se-containing protein subunit.

Since the Se-containing subunits and the chromatographic forms were derived from the same native Se-containing proteins, the distribution of a subunit and a chromatographic form arising from the same native protein should have been highly correlated. Therefore, the concentrations of Se in the subunit size groups were regressed one group at a time against the concentrations of Se in the chromatographic form groups (and vice versa) across all nine tissues by the technique of multiple linear regression [59] (two animals, $N = 18$). This analysis revealed the presence of 22 significant correlations between subunit size groups and chromatographic form groups ($p < 0.05$). This represents an upper limit to the number of uniquely different Se-containing proteins in this study since each native selenoprotein could have given rise to more than one correlation. This was probably the case for glutathione peroxidase, which gave rise to three chromatograhpic forms on DEAE Sephacel in Triton X-100 and urea. On the other hand, since there were seven different subunit sizes, plus the 26,500M_r, non-glutathione peroxidase, Se-containing subunit in plasma, there must have been at least eight different Se-containing polypeptides. Work is presently under way in this laboratory to separate all of the Se-containing proteins by HPLC to allow an accurate count of the total number of unique selenocysteine-containing proteins in the rat.

Since approximately two-thirds of the whole-body Se in rats is in non-glutathione peroxidase, selenocysteine-containing proteins, it is reasonable to expect that there may be other important biochemical activities associated with these proteins. Several other investigators [42, 50, 60] have also come to the conclusion that there must be biological functions of Se other than in glutathione peroxidase. It would seem, therefore, that a complete understanding of the powerful and diverse biological effects of dietary Se in animals will depend on discovering the biological or enzymatic activities of these other selenocysteine-containing proteins.

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